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Protective effect of D-glucose, L-leucine and fetal calf serum against oxidative stress in neonatal pancreatic islets

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Abstract

B-cell destruction during the onset of diabetes mellitus is associated with oxidative stress. In this work, we investigated the mechanisms of defense against oxidative stress present in neonatal islets and their modulation by D-glucose, L-leucine and fetal calf serum (FCS). Culturing neonatal rat islets in the presence of low D-glucose concentrations (2.8–5.6 mmol/l) and 1 mmol/l H_2O_2 increased the D-glucose uptake by islets sixfold compared to control levels. This effect was dose-dependently inhibited by D-glucose or FCS and by high concentrations of L-leucine. These supplements allowed islets to increase cytoplasmic catalase (CAT) activity only in response to H_2O_2, with no decrease in NO formation. Although L-leucine increased CAT activity and restored D-glucose uptake, it did not prevent damage to the islets. These data indicate that the most important H_2O_2 scavenger system in the islets is CAT and that this system can be modulated by metabolic substrates.

Keywords: Neonatal pancreatic islet; D-Glucose; L-Leucine; Fetal calf serum; Oxidative stress; H_2O_2; Catalase

1. Introduction

Pancreatic B-cells have low expression of reactive oxygen species (ROS) scavenger enzymes such as superoxide dismutase (SOD), catalase (CAT) and gluthathione peroxidase (GPx) [1], and oxidative stress in these cells is common [2]. The onset of type 1 diabetes is partly mediated by H_2O_2 released from activated lymphocytes that infiltrate islets [3–5]. In contrast, type 2 diabetes evolves to advanced clinical complications through islet damage and loss of insulin secretion, mediated by gluco-lipo-toxicity, TNFα cytotoxicity, and recognition of advanced glycated ends (AGEs) by islet resident macrophages and their subsequent activation [6–8].

Oxidative stress in B-cells occurs through different pathways [9]. Since B-cells express CuZnSOD (cytoplasm) and MnSOD (mitochondrial matrix) [10], they decompose ROS into H_2O_2 [11] but are unable to remove H_2O_2 because they lack a powerful peroxidase activity. Thus, H_2O_2 may accumulate in the cytoplasm and inhibit its own diffusion from the mitochondrial matrix [12]. Within the matrix, H_2O_2 causes lipid peroxidation [9] and provokes the cross-linking of thiol residues of inner mitochondrial membrane (IMM) and matrix proteins. Following oxidation, the ADP/ATP translocator, the major component of IMM proteins, interacts with proteins such as the voltage-dependent anion channel (VDAC) of the external mitochondrial membrane (EMM), cyclophilin D from the matrix, and ER proteins of the Bax and Bcl-2 families [12]. They form membrane permeability transition pores (MPTP) responsible for the decrease in IMM voltage, mitochondrial swelling and respiratory inefficiency [13].

In addition, cytoplasmic indicators of oxidative stress such as NIK and MEKK1 activate the transcription of iNOS and apoptotic genes [16–18]. iNOS uses NADPH, O_2 and L-arginine to increase NO production several fold [16,19], which in turn down-regulates the glycolytic and citric acid pathways [20,21], decreases cytochrome *c* oxidase complex

Abbreviations: SOD, superoxide dismutase; CAT, catalase; GPx, gluthathione peroxidase; TPx, thioredoxin peroxidase; BSA, bovine serum albumin; EDTA, ethylenediamine tetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; DTT, dithiothreithiol

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(COX) free-eletron scavenger activity [22,23] and alters nuclear signaling by increasing DNA exposure [24,25].

Enhanced generation of H_2O_2 and ROS in islets stimulates the transcription of apoptotic genes that results in mitochondrial and nuclear DNA fragmentation, inhibition of protein synthesis and reduction in islet cell mass. All of these processes can be prevented by fetal calf serum (FCS) or its components such as IGF-1, IGF-2 and GH [26,27].

In this work, we analyzed the effect of chronic exposure to D-glucose, L-leucine and FCS on the H_2O_2 scavenging system of neonatal rat islets. Our results indicate that all three components increase the rate of H_2O_2 degradation when H_2O_2 is present in culture media. Almost all the peroxidase activity detected was due to CAT. None of these substances reduced NO formation by islets exposed to H_2O_2. Finally, D-glucose and FCS, but not L-leucine, protected the islets against exposure to H_2O_2.

2. Material and methods

2.1. Islets isolation and culture

Neonatal (1–2 days old) and adult (4–6 months old) Wistar rats were purchased from the State University of Campinas animal facilities. After decapitation, neonatal [28] and adult [29] islets were isolated by collagenase (EC 3.4.24.3) digestion of pancreata in Hanks balanced salt solution. Islets were handpicked under a microscope, washed twice in sterile Hanks solution and cultured in RPMI 1640 medium (Sigma) supplemented with 2 g $NaHCO_3$/l and 1% penicillin/streptomycin, pH 7.4, and other supplements as indicated in the legends. Approximately 1000 islets/dish were maintained at 37 °C in a humidified atmosphere with 3% CO_2 for different periods of time. The medium was renewed every 48 h, as required.

2.2. NO production

NO formation was quantified by measuring the nitrite content of islet extracts. The islets in each dish were transferred to Eppendorf tubes containing 100 μl of Hanks solution (4 °C) and sonicated. The NO_2^- concentrations of total extracts were measured colorimetrically using a modified Griess reagent (1 part of 15 mg of sulfanilamide/ml in pure H_3PO_4 + 1 part of 1.5 mg NEED/ml in H_2O), using $NaNO_2$ dissolved in Hanks solution as standard. Absorbances were calculated as the difference between the readings at 540 and 600 nm. Assays were done immediately after recovery the islets from the dishes. NO_2^- levels were expressed as $[NO_2^-]$/[total protein] ratio.

2.3. D-Glucose uptake

D-Glucose concentrations in the media were determined using a commercial glucose enzymatic kit (Biotrol Diagnostic, Chennevières-lès-Louvres, France). D-Glucose uptake by the islets was measured as the variation in D-glucose concentrations in 10 ml of medium containing 5.6 mmol of D-glucose/l + 5% FCS at the end of 12 h in culture. Samples for D-glucose measurements were taken after filtration of the media through 0.45-μm pore filters (Millipore) to remove cells. The results were expressed as the D-glucose consumed/total protein content of islets in each dish. Extracts were prepared in Hanks solution by mechanical high-speed disruption of islets, at the end of the culture.

2.4. Peroxidase activity

Islet extracts were centrifuged at $10\,000g \times 6$ min to remove cellular membranes and organelles. The protein concentration of the liquid phase was measured (Biorad) and adjusted to 100 μg/ml by adding Hanks solution. H_2O_2 was added to each sample to a final concentration of 2.3 mmol/l and its concentration was then measured 0, 5, 10, 20, 30, 40 and 60 min later using a colorimetric reaction (Glucose Enzymatic Colour Liquide), against an H_2O_2 standard curve. The values were fitted to single exponential decay curves using the expression $[H_2O_2](t)=[H_2O_2]_{t=0} \times e^{-kt}$. The peroxidase activity of each sample was expressed as the k value (decay constant) obtained with given protein concentrations (100 μg/ml). Since the experiments with pure CAT showed a nonlinear crescent relationship between enzyme concentration and peroxidase activity, activities were not normalized.

2.5. Enzymatic kinetics

The velocity of H_2O_2 decomposition by islet protein or pure CAT (EC 1. 11.1.6, H_2O_2 oxidoreductase) (Sigma) was determined in Eppendorf tubes containing either islet protein or CAT dissolved is Hanks solution alone with increasing concentrations of H_2O_2 (0 to 300 mmol/l). Aliquots of the medium were taken 1, 2, 3 and 4 min after H_2O_2 addition, diluted with acidified Hanks solution (pH 1.0) and assayed for H_2O_2, as stated. Initial H_2O_2 concentrations and the degradation rate were determined by linear regression over the data. K_m values were calculated from Boltzman sigmoidal curves fitted to the points.

2.6. Statistical analysis

The results were expressed as the means ± S.E. and the significance of differences was assessed by Student's t test. A value of $P<0.05$ indicated significance.

3. Results

Peroxidase activity in neonatal islets was approximately three times greater than adult islets ($k = 0.111 \pm 0.007$ min^{-1} vs. $k = 0.034 \pm 0.004$ min^{-1}, respectively, at 100 μg protein/

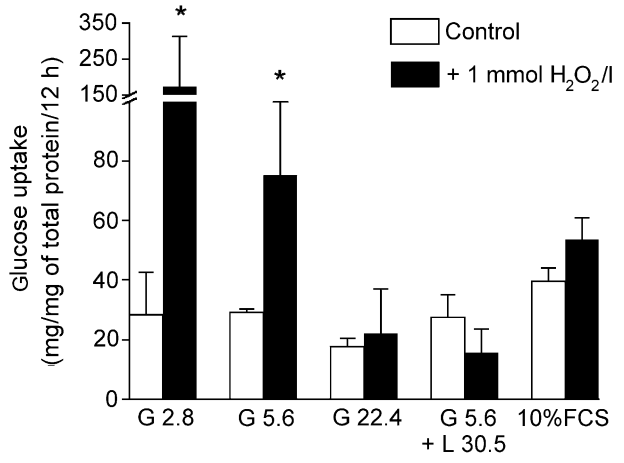

Fig. 1. Effect of H_2O_2 on D-glucose use by cultured neonatal islets. Islets were cultured for 4 days in RPMI medium with or without 1 mmol of H_2O_2/l and different concentrations of D-glucose (G; 2.8, 5.6 and 22.4 mmol/l) + 5% FCS, L-leucine (L; 30.5 mmol/l) + 5.6 mmol of D-glucose/l and 5% FCS or FCS (10%) + 5.6 mmol D-glucose/l. This medium was subsequently replaced by one containing 5.6 mmol of D-glucose/l and 5% FCS. The bars represent cumulative D-glucose use for 12 h as described in Section 2. The values are the means ± S.E. of six independent experiments. * $P < 0.05$ vs. the respective control.

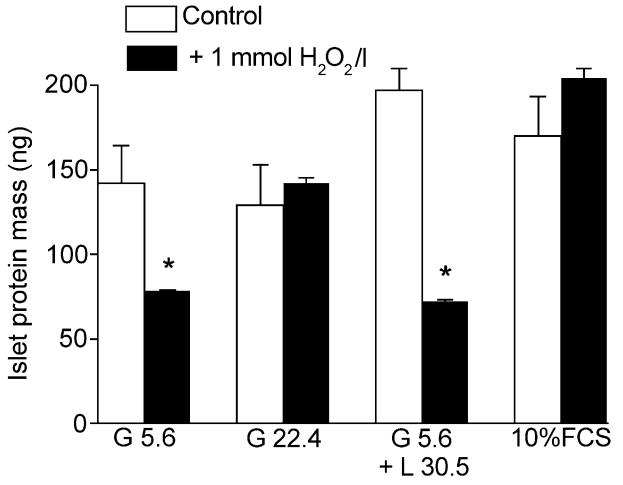

Fig. 3. Effect of H_2O_2 on protein mass of cultured neonatal islets. Islets were cultured for 4 days in RPMI medium with or without 1 mmol of H_2O_2/l and different concentrations of D-glucose (G; 5.6 and 22.4 mmol/l) + 5% FCS, L-leucine (L; 30.5 mmol/l) + 5.6 mmol of D-glucose and 5% FCS or FCS (10%) + 5.6 mmol of D-glucose/l. Subsequently, groups of 200 islets were sonicated in 100 μl of Hanks solution and the protein concentration was measured by a colorimetric assay. The bars represent the means ± S.E. of six independent experiments. * $P < 0.05$ vs. the respective control.

ml, $n = 4$; $P < 0.05$). This activity was almost attributed to hydrophilic proteins since the removal of membranes and organelles from islet extracts did not alter the ability to decompose H_2O_2 ($k = 0.24 \pm 0.02$ min^{-1} for the liquid phase vs. $k = 0.22 \pm 0.02$ min^{-1} for the total extract, at 200 μg protein/ml, $n = 4$; NS). Heating the extracts at 100 °C for 5 min reduced peroxidase activity to less than 3% of normal values, indicating that heat-denatured proteins are responsible for H_2O_2 degradation.

The presence of 1 mmol H_2O_2/l in culture medium containing low concentrations of D-glucose (2.8 mmol/l) dramatically increased the uptake of this sugar by neonatal islets (Fig. 1). This effect was significantly reduced by physiological concentrations of D-glucose (5.6 mmol/l)

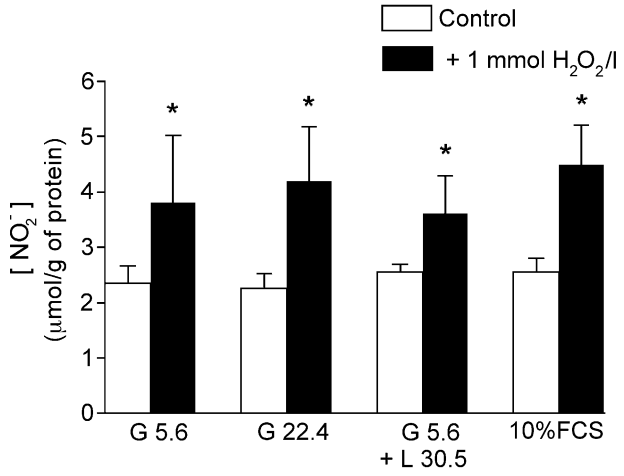

Fig. 2. Effect of H_2O_2 on nitrite production by cultured neonatal islets. Islets were cultured for 4 days in RPMI with or without 1 mmol of H_2O_2/l and different concentrations of D-glucose (G; 2.8, 5.6 and 22.4 mmol/l) + 5% FCS, L-leucine (L; 30.5 mmol/l) + 5.6 mmol of D-glucose/l and 5% FCS or FCS (10%) + 5.6 mmol of D-glucose/l. After culture, the islets were sonicated and NO_2^- concentrations within the cells were measured as described in Section 2. The values are the means ± S.E. of six independent experiments. * $P < 0.05$ vs. the respective control.

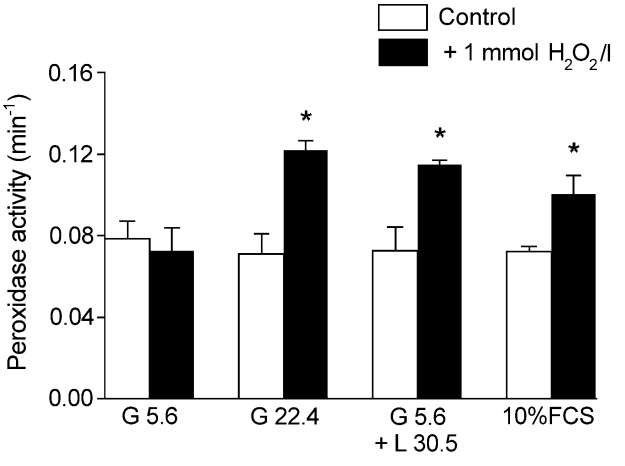

Fig. 4. Effect of H_2O_2 on peroxidase activity of cultured neonatal islets. Islets were cultured for 4 days in RPMI medium with or without 1 mmol H_2O_2/l and different concentrations of D-glucose (G; 2.8, 5.6 and 22.4 mmol/l) + 5% FCS, L-leucine (L; 30.5 mmol/l) + 5.6 mmol of D-glucose/l and 5% FCS or FCS (10%) + 5.6 mmol of D-glucose/l. The islets were subsequently sonicated and the peroxidase activity in the extract was measured as described in Section 2. The bars are the means ± S.E. of 10 independent experiments. * $P < 0.05$ vs. the respective control.

Table 1
Effects of different concentrations of D-glucose and FCS on the peroxidase activity of islet extracts

Agents		Peroxidase activity (%)
D-Glucose (mmol/l)	FCS (%)	
2.8	5	116 ± 9
5.6	5	100
11.2	5	110 ± 30
22.4	5	70 ± 20 *
5.6	2	130 ± 6 *
5.6	5	100
5.6	7	85.4 ± 0.1 *
5.6	10	60 ± 20 *

The values are the means ± S.E. of four experiments.
* $P<0.05$ compared to the respective controls (5.6 mmol/l for D-glucose and 5% for FCS).

and abolished by 22.4 mmol of D-glucose/l, by the combination of D-glucose (5.6 mmol/l) plus L-leucine (30.5 mmol/l) and by 10% FCS (Fig. 1). H_2O_2 per se did not affect the D-glucose concentration in the incubation medium.

Exposure to 1 mmol of H_2O_2/l also increased nitrite production by the islets. This occurred even in the presence of D-glucose, L-leucine or FCS (Fig. 2). There was no correlation between D-glucose uptake and nitrite production, indicating that NO formation may not be responsible for increased D-glucose uptake by islets exposed to H_2O_2.

Culturing the islets in the presence of 1 mmol of H_2O_2/l and 5.6 mmol of D-glucose/l reduced the islet mass by 50% compared to the controls (absence of H_2O_2). High D-glucose (22.4 mmol/l) or FCS (10%), but not L-leucine (30.5 mmol/l), protected islet against damage by H_2O_2 (Fig. 3). Fig. 4 shows that the peroxidase activity of islet extracts was significantly increased by the exposure to H_2O_2, however, this increased activity was dependent on the presence of high D-glucose, L-leucine or FCS.

Unexpectedly, incubation of islets with high D-glucose and FCS concentrations in the absence of H_2O_2 dose-dependently reduced the peroxidase activity of islet extracts (Table 1).

In the next series of experiments, we investigated the enzyme responsible for H_2O_2 degradation in islet extracts. Fig. 5A shows that the enzyme activity of the extract did not change during successive challenges with H_2O_2, and that the incubation of samples with thiol reductants such as L-cysteine (1 mmol/l), 2-mercaptoethanol (1 mmol/l) or dithiothreithiol (DTT) (0.5 mmol/l) between two H_2O_2 additions did not affect the peroxidase activity of the extracts. Fig. 5B illustrates that addition of different inhib-

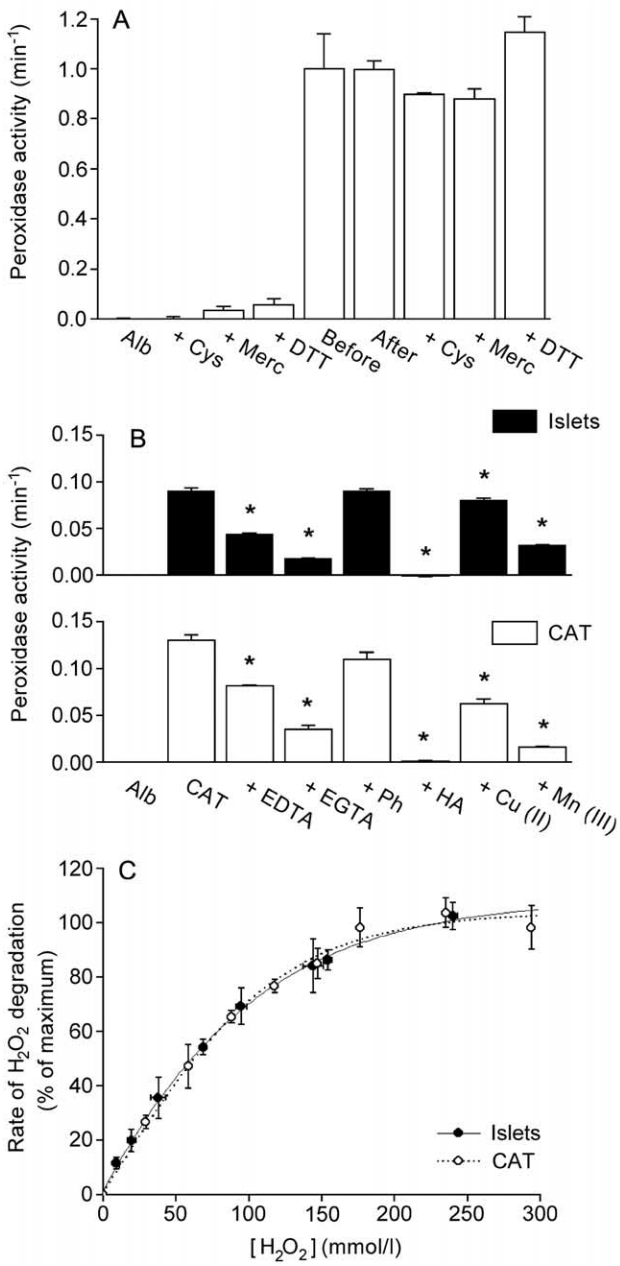

Fig. 5. Nature of the peroxidase activity in pancreatic neonatal islets. Islets were cultured in RPMI medium containing 5.6 mmol of D-glucose/l and 5% FCS. (A) represents the peroxidase activity of islet extracts (200 μg of protein/ml), the first challenge with H_2O_2 (before) and at the second challenge after a 30-min incubation with the following substances at the indicated final concentrations (pH adjusted to 7.4) in Hanks solution: none (After), 1 mmol of L-cysteine/l (Cys), 1 mmol of 2-mercaptoethanol/l (Merc) and 0.5 mmol of DTT/l (DTT). Negative controls were BSA (Alb; 100 μg/ml) without or with the same additions. The values are the means ± S.E. of six independent experiments. (B) (upper panel) represents the peroxidase activity of islet extracts (500 μg of protein/ml) in the presence of EDTA (40 mmol/l), EGTA (40 mmol/l), phenantroline (Ph) (10 mmol/l), hydroxylamine (HA) (10 mmol/l), and saturated $CuCO_3$ and $Mn_2(CO_3)_3$ (pH adjusted to 7.4). BSA (Alb) (100 μg/ml) was used as negative control. Identical experiments were done with purified catalase (lower panel). The values are the means ± S.E. of six independent experiments. * $P<0.05$ vs. control values (no additions). (C) represents the rate of H_2O_2 degradation measured at increasing concentrations of H_2O_2 using islet extracts or purified catalase (5.4 μg/ml). The K_m for the islet extracts and purified catalase were 70 ± 1 and 70 ± 0.8 mmol/l, respectively ($n=5$).

itors of CAT similarly reduced the peroxidase activity of islet extracts (upper panel) and of purified CAT (lower panel). Finally, the rate of H_2O_2 degradation for islet extracts and purified CAT had identical kinetics with K_m close to 70 mmol/l (Fig. 5C). The optimal pH for the islet extracts and purified CAT was 8.5.

4. Discussion

The best known ROS scavenging system in pancreatic islets consists of: COX, which avoids the leakage of free electrons from the respiratory chain [23]; SOD isoforms, which convert ROS into H_2O_2 in the cytoplasm or inside mitochondria; glutathione/glutathione reductase, which uses NAD(P)H as an electron source to regenerate glutathione within mitochondria; and CAT, which decomposes H_2O_2 into $H_2O + 1/2\ O_2$ [1]. We have also recently observed the presence of thioredoxin peroxidase (TPx) in islets that may represent another system for cytoplasmic H_2O_2 degradation (unpublished data).

As shown here, neonatal islets were protected better against oxidative stress than adult islets because of their more active peroxidase system. This greater protective ability against oxidative stress may be linked to higher circulating levels of growth factors in neonatal rats, thus allowing the islets to increase the rate of H_2O_2 degradation, if H_2O_2 is generated in large amounts (Fig. 4). Since this peroxidase system is almost totally localized in the water-soluble cell fraction, it is likely to be different from the peroxidase system of mitochondrial membranes [14].

Oxidative stress induced by H_2O_2 uncouples mitochondria by forming MPTP [14]. Thus, islets exposed chronically to H_2O_2 would be expected to metabolize higher amounts of D-glucose than controls in order to generate similar quantities of ATP, even at a nonstimulatory D-glucose concentration. In the presence of stimulatory concentrations, more ATP would be required for synthesis, maturation and translocation of insulin to the membrane [30]. The addition of D-glucose, L-leucine or FCS to the media containing H_2O_2 enhanced H_2O_2 degradation within the islets, thus decreasing the metabolic demand for ATP synthesis (Figs. 1 and 4).

NO has recently been associated with a reduction in COX activity that could increase ROS and H_2O_2 formation [23]. NO also reacts with ROS or H_2O_2 generated in mitochondria to form $ONOO^-$, an even more active radical [21]. Oxidative stress causes IkB heterodimer phosphorylation, subsequent NF-kB translocation to the nucleus and transcription of iNOS, in a positive feedback mechanism [17,31]. Since D-glucose, L-leucine and FCS did not decrease NO production in islets, with or without H_2O_2 in the culture media (Fig. 2), they probably failed to reduce iNOS expression. However, even with greater NO production, glucose uptake by islets was normalized in the presence of high concentrations of D-glucose, L-leucine and FCS (Fig. 1), indicating that increased NO production was not sufficient to induce mitochondria damage [21,32,33].

Thiol-reducing enzymes probably participate strong in H_2O_2 degradation by accelerating the reaction $2R\text{-}SH + H_2O_2 \Rightarrow RSSR + 2H_2O$ [15]. These enzymes and cofactors such as glutathione are reduced to the R-SH form by small thiol molecules such as L-cysteine, 2-mercaptoethanol and DTT [34]. However, treatment of samples with these thiol groups after exposure to H_2O_2 did not alter peroxidase activity, indicating that at the high concentrations of H_2O_2 used in our experiments [4], these enzymes and cofactors have a marginal participation in the degradation of H_2O_2 (Fig. 5A). To investigate the nature of peroxidase activity in islets, we used nonspecific inhibitors of metal-dependent enzymes such as ethylenediamine tetraacetic acid (EDTA), ethylenediamine tetraacetic acid (EGTA), Cu^{2+}, Mn^{3+}, phenantroline and hydroxylamine [35,36]. The variations in the peroxidase activity of islet extracts with each inhibitor mimicked the variations seen with purified CAT. These results, which were confirmed by the enzyme kinetics, strong indicated that this peroxidase was CAT (Fig. 5B,C).

Antioxidant systems must be regenerated by electron donors such as NAD(P)H, $FADH_2$, glutathione, DTT or other thiol groups from soluble (e.g. thioredoxin) or membrane-bound proteins (EMM) [15,34]. In normal cells, the concentration of H_2O_2 is in the range of 1 μmol/l, and CAT has to be coupled to NADPH to be active. In contrast, in the range of 1 mmol of H_2O_2/l, as used in our experiments and usually found in lymphocyte-infiltrated islets [3], CAT no longer requires NADPH [37]. NADPH levels, kept elevated by metabolic substrates (D-glucose and L-leucine), reduced the peroxidase activity in islets (Table 1). Thus, the decreasing amount of CAT seen in the presence of increasing concentrations of D-glucose or FCS may correlate with an increase in its individual molecules activity [37,38].

The increase in peroxidase activity produced by FCS suggests another possible mechanism in the regulation of scavenging systems. FCS is known to preserve islets by suppressing apoptotic genes and stimulating the transcription of anti-apoptotic ones [39]. This is the case of some Bcl-2 family proteins that inhibit the formation of MPTP [27]. Proteins with increased expression in response to growth factors may exert other protective effects against oxidative stress that cannot be explained simply by increasing CAT activity (Figs. 3 and 4). Our data indicate that, in the case of severe oxidative stress induced by H_2O_2 in islets, high concentrations of D-glucose, but not L-leucine (which shares the mitochondrial part of the metabolic pathway with D-glucose), may exert an FCS-like protection through some as yet unknown mechanism [38,40].

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